EXHIBIT A

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Generation of protein-reactive antibodies by short peptides is an event of high frequency: Implications for the structural basis of immune recognition

(monoclonal antibodies/transforming protein/protein folding/predetermined sequence specificity/v-*fes*)

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ABSTRACT Recent studies have shown that chemically synthesized small peptides can induce antibodies that often react with intact proteins regardless of their position in the folded molecule. These findings are difficult to explain in view of the experimental and theoretical data which suggest that in the absence of forces provided by the folded protein, small peptides in aqueous solution do not readily adopt stable structures. In order to rationalize the two findings, there has been general acceptance of a stochastic model which suggests that the multiple conformers of a peptide in solution induce sets of antibodies with a small percentage reactive with conformations shared by the folded protein. This stochastic model has become less tenable as the success rate for the generation of protein-reactive anti-peptide antibodies has grown. To test the stochastic model, we have used monoclonal anti-peptide antibodies as a way of estimating the frequency with which small peptides induce antibodies that react with folded proteins. We have made monoclonal antibodies to six chemically synthesized peptides from three proteins. The frequency with which the peptides induce protein-reactive antibodies is at least 4 orders of magnitude greater than expected from previous experimental work and vastly different from what would be predicted by calculating the possible number of peptide conformers in solution. These findings make the stochastic model less likely and lead to consideration of other models. Aside from their practical significance for generation of highly specific reagents, these findings may have important implications for the protein folding problem.

Recent studies have shown that antibodies to short peptides often cross-react with intact proteins irrespective of where the peptide is located on the surface of the intact structure (reviewed in refs. 1 and 2). The initial assumption was that these peptides adopted many conformations in solution, occasionally assuming one that approximated its cognate structure in the native molecule (3). Thus, an anti-peptide antiserum could be thought of as a collection of antibodies to different conformations, with some percentage reactive with conformations shared by the folded protein. However, the problem with this assumption is that in the absence of forces engendered by neighboring structures, peptides are thought to have a vast number of conformations, and one would predict a relatively low success rate of generating antibodies reactive with intact proteins when a large number of different peptides are tried. This prediction turns out not to be true because a large number of different protein-reactive anti-peptide antibodies have been generated in the last 2 yr (1–3). There are alternative explanations that could explain the high rate of success with which short peptides induce protein-reactive antibodies. The favored conformation of a peptide in solution might approximate the one it takes in the folded protein, or antibodies could induce or stabilize conformations in otherwise flexible peptides or proteins. Before one focuses on these alternatives, it is important to measure what percentage of time a peptide in solution elicits antibodies reactive with the intact protein. This cannot be measured by studying a polyclonal immune response because the diversity of the immune response may allow low-frequency events to be scored the same as higher-frequency events. To overcome this problem, we have studied monoclonal anti-peptide antibodies. Monoclonal antibodies to six short peptides from three proteins were prepared. Between 25% and 100% of the antibodies bound to the intact protein, even though they initially were selected only for their ability to bind to free peptides. These results allow consideration of models of immune recognition in which conformational freedom of peptides is constrained or local structure in the intact protein is relaxed.

MATERIALS AND METHODS

Viruses and Cell Lines. X47, A/Aichi, A/Swine, and B/Hong Kong viruses were grown in chicken eggs and purified from allantoic fluid. The Snyder–Theilen strain of feline sarcoma virus (ST-FeSV) was grown in MSTF cells, a productively transformed mink cell line infected with the ST strain of FeSV and FeLV-B (4). Histocompatibility antigens HLA-DR1 and -2 were from the homozygous lymphoid B-cell lines LG-2 and GM3107, respectively (5).

Synthesis of Peptides. Peptides were synthesized by using solid-phase methods (6). The composition of each peptide was confirmed by amino acid analysis. Peptides were synthesized according to the amino acid sequence or were predicted from the nucleotide sequence: X47 hemagglutinin 1 (HA1) (7), Snyder–Theilen FeSV v-*fes* (8), HLA-DR1 α-chain and HLA-DR1 β chain (5), and HLA-DR2 β chain (9).

Coupling of Synthetic Peptides to Carrier Protein. Synthetic peptides were coupled to the carrier protein keyhole limpet hemocyanin (KLH) through the cysteine of the peptide, with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as the coupling reagent as described (10). In the case of the X47 peptide, the presence of two cysteines allowed a higher variability of coupling, including peptides joined to the carrier by both cysteines.

Immunization and Fusion. Peptides of HA1 and v-*fes* coupled to KLH were used to immunize 129 GIX$^+$ mice as described (11). Spleen cells were fused with SP2/0 myeloma cells with polyethylene glycol 1500 (Baker); resuspended in 400 ml

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Abbreviations: ST-FeSV, Snyder–Theilen strain of feline sarcoma virus; KLH, keyhole limpet hemocyanin; HA, hemagglutinin.

of Dulbecco's high-glucose minimal essential medium containing 10% fetal calf serum, 100 μM hypoxanthine, 1.0 μM methotrexate, and 16 μM thymidine; plated into 30 microtiter plates; and grown as described (11). Peptides of HLA-DR α and β chains coupled to KLH were used to immunize BALB/c mice and were fused to P3/X63.Ag8 myeloma cells. These fused cells were treated as described above.

Antibody Binding Assay. Hybridomas producing anti-peptide antibodies were detected with an enzyme-linked immunosorbant assay (ELISA) method as described (12). Briefly, approximately 50 pmol of peptide was dried onto microtiter plates, fixed by methanol, and incubated with tissue culture supernatant. After the hybridoma antibody was thoroughly washed, binding was detected by using rabbit anti-mouse κ chain (Litton), followed by a glucose oxidase-conjugated goat anti-rabbit antiserum. Binding was visualized with ABTS dye (Boehringer Mannheim) in the presence of glucose and horseradish peroxidase (12). Isotype was determined by substituting various rabbit anti-mouse λ or heavy chain sera for the anti-mouse κ chain as described above.

Immunoprecipitation of HA1. X47 virus, pelleted from allantoic fluid, was radioiodinated as described (13) and subjected to immunoprecipitation (14).

Electrophoretic Transfer and Immunological Detection of Proteins in Nitrocellulose. Allantoic fluid, glycoprotein preparations, or cell extracts were subjected to polyacrylamide electrophoresis. The protein was transferred to nitrocellulose (Schleicher & Schuell), and peroxidase-labeled rabbit anti-mouse IgG (Tago) diluted 1:1,000 was incubated with the transfers for 1 hr at 25°C followed by washes as described (14). The bound antibody was visualized by incubation in 10 mM Tris, pH 7.4/0.009% H_2O_2/0.0025% 3,3'-dimethoxybenzidine dihydrochloride (Kodak).

RESULTS

A High Percentage of Monoclonal Antibodies That Bind to Peptides Recognize the Intact Molecule. Monoclonal antibodies with predetermined sequence specificity were generated by hyperimmunizing mice with synthetic peptides coupled to KLH. A synthetic peptide of 36 amino acids representing residues 76–111 of X47 HA1 (10) was chosen to estimate the frequency with which a free peptide shares conformation with its cognate structure in the intact protein. The 36-mer chosen for this study had been shown previously to produce in rabbits high titers of antibody that would react with the intact HA molecule (10). After the third injection, hybridomas were generated, and the tissue culture supernatant from the various lines was tested for antibody that would bind to the free peptide, which had been dried and fixed onto the bottom of microtiter test plates. A single fusion experiment with one spleen produced 21 cell lines that secreted stable antibody reactive with the synthetic 36-mer peptide. These lines are listed in Table 1 in order of the relative strength of the antibody binding to the peptide. Also listed is the relative binding to the X47 virus, which had been dried to microtiter plates. Of the 21 cell lines that synthesized antibody that readily bound to the synthetic peptide, 16 produced antibody that bound significantly to the virus.

Recognition of a Determinant Is Not Dependent on the Mode of Antigen Presentation. To verify that the binding activity for the virus dried onto plates was specific for the HA molecule, virus was labeled with ^{125}I and subjected to immunoprecipitation with tissue culture supernatant from the various hybridoma cell lines. Fig. 1 shows the immunoprecipitation patterns of all 21 cell lines. The complex precipitated from the mixture contains the HA1 chain of the HA molecule, and the efficiency of precipitation agrees well with the plate binding data. The five cell lines that did not react well with the virus dried onto microtiter plates also did not immunoprecipitate significantly the HA molecule (lanes F, L, O, T, and U). Four of the hybridomas that reacted weakly with the virus dried onto plates also poorly immunoprecipitated the HA molecule (lanes K, M, P, and R). The remaining 12 hybridomas readily precipitated the HA molecule, indicating that the antibodies could recognize the intact HA1 (in a solution of phosphate-buffered saline) as well as HA dried and methanol-fixed onto microtiter plates. In addition, the antibodies were tested for their ability to recognize HA1 bound to nitrocellulose sheets after electrophoretic transfer from a polyacrylamide gel. Again, there was an excel-

Table 1. Cell lines producing X47 peptide-reactive antibodies

Hybridoma	Isotype	Peptide binding, OD_{414}	Virus binding, OD_{414}	Ratio, virus/peptide
H23D02	2b	690	480	0.70
H26D08	2b	684	361	0.53
H26A09	2a	672	414	0.62
H16E07	1	670	439	0.66
H24E07	2a	666	358	0.54
H22F05	2a	662	53	0.08
H19B10	2b	621	375	0.60
H20C01	2a	616	340	0.55
H19D03	2a	597	561	0.94
H21C08	2b	578	440	0.76
H26F02	1	561	201	0.36
H17D12	1	544	112	0.21
H23F10	2a	450	267	0.59
H20F04	2a	428	357	0.83
H17D09	2a	415	6	0.01
H26D02	2a	413	143	0.35
H22F02	2a	405	374	0.92
H17B11	1	394	165	0.42
H23B05	1	368	254	0.69
H16C08	1	333	−9	−0.03
H20E03	1	322	34	0.11

Cell lines were named as described (11) and their isotype was determined. All isotypes were κ for light chain and IgG for heavy chain. Binding to peptide was proportional to the optical density measured at 414 nm. Values listed are 1,000 times the average of two tests. Peptide was tested at 50 and 100 pmol per well, whereas virus was tested at 1:5 and 1:10 dilution of allantoic fluid. An average background (media control) of 57 was subtracted from virus readings.

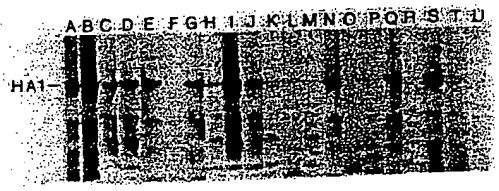

FIG. 1. Immunoprecipitation of X47 HA1. X47 virus was isolated from allantoic fluid by ultracentrifugation at 100,000 × *g*. The virus pellet was labeled with ^{125}I. The labeled virus was incubated with 25 μl of tissue culture supernatant from the following cell lines: H23D02 (lane A), H26D08 (lane B), H26A09 (lane C), H16E07 (lane D), H24E07 (lane E), H22F05 (lane F), H19B10 (lane G), H20C01 (lane H), H19D03 (lane I), H21C08 (lane J), H26F02 (lane K), H17D12 (lane L), H23F10 (lane M), H20F04 (lane N), H17D09 (lane O), H26D02 (lane P), H22F02 (lane Q), H17B11 (lane R), H23B05 (lane S), H16C08 (lane T), and H20E03 (lane U). Precipitates were collected with fixed *Staphyloccocus aureus* and subjected to electrophoresis on a 5–17% $NaDodSO_4$/polyacrylamide gel prior to autoradiography. The figure is a composite of three gels and, thus, differences in mobility of the faster migrating bands are not significant and are the result of viral proteins that bind to each other during precipitation. These bands do not appear when proteins are separated prior to reacting with antibody (see Fig. 2).

lent correlation for the ability to bind to the intact molecule under various conditions of presentation. All 12 of the antibodies that recognized the intact molecule in solution or HA when methanol-fixed to microtiter plates also recognized HA1 bound to nitrocellulose sheets (see Fig. 2 for representative examples). In addition, the four hybridomas H26F02, H23F10, H26D02, and H17B11, which reacted weakly with the virus dried onto the microtiter plates and poorly with HA in solution, did react, however, with the HA1 molecule when bound to nitrocellulose (data not shown).

Hybridomas Recognize Different Determinants. It was important to rule out the possibility that we had isolated the progeny of only a few clones ("Jackpot"). To elucidate different binding specificities, the panel of monoclonal antibodies was allowed to react with four strains of influenza virus. X47, the virus from which the sequence for the peptide was derived, is classified as an H_3N_2 strain as is A/Aichi, which differs from X47 in only two residues within the sequence of the peptide. Both changes are at the amino-terminal end of the peptide—i.e., residue 79 changes from glycine to valine and residue 84 changes from lysine to threonine. The latter change creates a potential glycosylation site in A/Aichi. The other two viruses, A/Swine and B/Hong Kong, differ extensively from X47 throughout the region of the peptide. Fig. 2 shows representative reactivities with HA1 bound to nitrocellulose sheets. H24B07 reacted well with both X47 and A/Aichi but not with A/Swine and B/Hong Kong. All other hybridomas that were reactive with HA1 on nitrocellulose sheets showed the same pattern except H19D03, which reacted very strongly with X47 but revealed only a minor reactivity band with A/Aichi, indicating that H19D03 recognized a unique determinant. The hybridomas could be further subdivided because of their reactivity with a set of 15 nested peptides (unpublished data). In brief, the reactivity pattern with the nested fragments showed that the panel of monoclonal antibodies recognize at least seven determinants.

High-Frequency Recognition of Intact Protein by Anti-Peptide Hybridomas Is a General Phenomenon. To insure that the results from the influenza system were not unique to the length or character of that peptide, monoclonal antibodies were produced against five additional peptides from two different proteins. Representative reactivities are shown in Fig. 3. Lanes 1 and 2 in Fig. 3 *Left* depict the detection of the M_r 85,000 polyprotein of the ST-FeSV encoding the v-*fes* gene. Both of the monoclonal antibodies that recognized the 30-mer listed in Table 2 also reacted with a carboxyl-terminal 12-mer (data not shown) and the intact molecule. Fig. 3 *Right* shows the pattern of reactivity with HLA-DR antigens of hybridomas produced against synthetic peptides of both HLA-DR α and β chains. The β chains of HLA-DR2 antigen were detected by monoclonal antibodies prepared both against a 14-mer, representing residues 61–73 of the HLA-DR2 β-chain sequence (lane 1), and a 15-mer, representing residues 60–73 of a HLA–DR β chain, whose sequence was determined along with that of the HLA-DR2 β chain (lane 2). The β chain of HLA-DR1 antigen also could be detected with a monoclonal antibody against a 16-mer representing residues 20–35 of the HLA-DR1 β-chain sequence (lane 3). In addition, a hybridoma prepared against residues 20–32 of the HLA-DR1 α-chain sequence reacted specifically with the α chain of HLA-DR1 antigen (lane 4). Table 2 summarizes all of the monoclonal antibodies used in this study. In each case, monoclonal antibodies could be generated that recognized both the synthetic peptide and the intact molecule. The number of hybridomas recognizing the intact molecule compared to the number binding to the peptide was 16/21, 2/2, 1/3, 3/6, 1/2, 1/4 for HA1, v-*fes*, HLA-DR1 α chain, HLA-DR1 β chain, and two sequences for HLA-DR2 β chain, respectively. These ratios indicate that sharing of immunological determinants between synthetic peptides and the intact molecule is a frequent event.

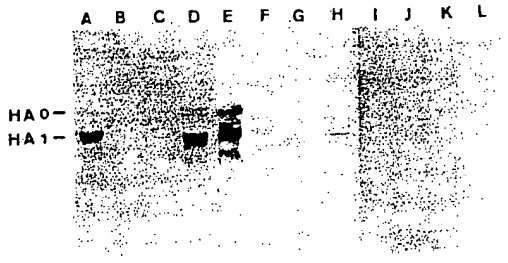

FIG. 2. Immunological detection of X47 and A/Aichi HA1 on nitrocellulose sheets. Allantoic fluid (50 μl) from eggs infected with X47, B/Hong Kong, A/Swine, or A/Aichi virus was separated by electrophoresis on a 5–17% $NaDodSO_4$/polyacrylamide gel prior to electrophoretic transfer to nitrocellulose: X47 (lanes A, E, and I), B/Hong Kong (lanes B, F, and J), A/Swine (lanes C, G, and K), and A/Aichi (lanes D, H, and L). The nitrocellulose sheets were incubated with tissue culture supernatant from H24E07 (lanes A–D), H19D03 (lanes E–H), or (as a control) $R_2$06B08, anti-Rauscher murine sarcoma virus gp70 hybridoma (ref. 11) (lanes I–L).

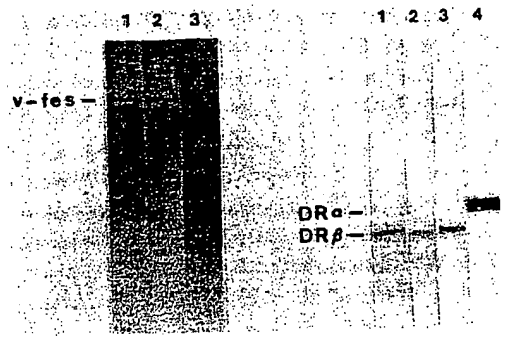

FIG. 3. Immunological detection of ST-FeSV v-*fes*, HLA-DR1 α chain, HLA-DR1 β chain, and HLA-DR2 β chain. (*Left*) Cell extracts of $\approx 10^5$ MSTF cells were electrophoresed onto a 15–17% polyacrylamide gel, transferred to nitrocelluose, and allowed to react with tissue culture supernatant S10F03 (lane 1), S22D06 (lane 2), and H19B10 (lane 3). (*Right*) Glycoprotein preparations (10 μg) purified from 10^5 GM3107 (lanes 1 and 2) or LG-2 (lanes 3 and 4) cells were enriched by affinity chromatography on lens culinaus lectin (5), subjected to electrophoresis on a 10% polyacrylamide gel, electrophoretically transferred to nitrocellulose sheets, and allowed to react with tissue culture supernatant KS2/1 prepared against HLA-DR2 β chain (residues 61–73) (lane 1), KS5/1 prepared against HLA-DR2 β chain (residues 60–73) (lane 2), KS3/1 prepared against HLA-DR1 β chain (residues 20–35) (lane 3), and KS4/1 prepared against HLA-DR1 α chain (5, 9) (lane 4).

DISCUSSION

The surprising observation that a large number of synthetic peptide antigens elicit antibodies that recognize the peptide in the intact protein (1–3) raises important questions about the structural basis for the generation of an immune response (immunogenicity) and for recognition by antibodies (antigenicity). The frequent ability of synthetic peptides to generate antibodies that recognize intact proteins could readily be reconciled if the synthetic peptide antigens could stably adopt the precise local structure of that sequence in the intact protein. Thus, one popular explanation for the success of synthetic peptide antigens in generating antibodies that recognize intact proteins involves a stochastic model in which the diversity of the immune response (and the nature of the screening procedures) allows the observation of an infrequent event—namely, the peptide

Table 2. Summary of monoclonal antibody reactivity with intact proteins

Protein*	Peptide†	Reactivity‡
X47 HA1	HCDGFQNEKWDLFVERSKAFSNCY-PYDVPDYASLRS (residues 76–111)	16/21
ST-FeSV v-*fes*	SDVWSFGILLWETFSLGASPYPNLS-NQQTR(C) (residues 703–732)	2/2
HLA-DR1 α chain	GEFMFDFDGDEIF(C) (residues 20–32)	1/3
HLA-DR1 β chain	GTERVRLLELFTYN(C) (residues 20–35)	3/6
HLA-DR2 β chain	WNSQKDILEQARA(C) (residues 61–73)	1/2
HLA-DR2 β chain	YWNSQMDFLEEARA(C) (residues 60–73)	1/4

* Amino acid sequence data predicted or determined previously: X47 HA1 (7), ST-FeSV v-*fes* (8), HLA DR1 α and β chains (5), and HLA DR2 β chain (9).

† Amino acid abbreviations are: A, alanine; G, glycine; T, threonine; C, cysteine; P, proline; M, methionine; V, valine; I, isoleucine; L, leucine; Y, tyrosine; F, phenylalanine; W, tryptophan; D, aspartic acid; E, glutamic acid; R, arginine; K, lysine; H, histidine; S, serine; N, asparagine; Q, glutamine; and (C), cysteine added for coupling purposes (not found or predicted by sequence). Numbers in parentheses represent the amino acid numbers of determined or predicted sequences.

‡ Number of hybridomas that react with the intact molecule electrophoretically transferred to nitrocellulose sheets relative to the number of hybridomas that react with the peptide in an enzyme-linked immunosorbant assay.

adopting the native conformer. However, the idea that short peptides (10–30 residues) frequently form stable structures is inconsistent with both theoretical (15) and experimental (16) studies, which suggest that although some short peptides (17–20) can adopt stable structures, the majority do not do so in aqueous solution. Instead, the peptides are thought to exist as an ensemble of a large number of transient conformational states in dynamic equilibrium. To test the stochastic model, a method is needed that estimates the frequency with which anti-peptide antibodies recognize native proteins. Monoclonal antibodies provide a convenient means to obtain such a frequency estimate. Because each monoclonal antibody is derived from a single cell that produces only one specificity, the ratio of the number of clones producing anti-peptide antibody that recognizes the intact protein molecule to the total number of clones producing antibody that binds the peptide itself provides a reasonable estimate of the true frequency, provided that the total number of isolates is sufficiently large and that the immune response is not inherently biased toward native conformation.

In the present study, a large percentage of the anti-peptide antibody-producing hybridomas secreted an antibody that recognized the intact protein molecules in Western blots. In the case of the antibodies to peptides of the influenza HA, the reactivity of the antibody to the intact protein was confirmed in enzyme-linked immunosorbant assays and by immunoprecipitation assay, suggesting that the recognition is independent of the mode of presentation of the intact protein. This is significant because the three assays involve different extents of denaturation of the intact protein. In all assay conditions, however, the degree of chain folding is greater than the random folding predicted by current theories of peptide structure.

These results suggest that sufficient structural information for high-frequency recognition of intact proteins is contained in peptides as small as 13 amino acid residues. Moreover, the frequencies observed are inconsistent with any stochastic model, which suggests that a *rare* native-like conformer of the peptide is responsible for generating the antibodies that recognize the intact protein.

Similar high-frequency recognition of native proteins by monoclonal antibodies raised against peptides also has been observed by others. Rohrschneider and his colleagues have made monoclonal antibodies to a 14-amino-acid peptide representative of positions 498–512 of the transforming protein of Prague C sarcoma virus. They found that 6 of 24 monoclonal antibodies reacted with the protein in transformed cells (L. R. Rohrschneider and L. E. Gentry, personal communication). Arnheiter *et al.* prepared monoclonal antibodies to a 56-amino-acid fragment of interferon (21). The binding of the monoclonal antibodies to 56-, 96-, and 33-residue fragments and to the complete α1 and α2 interferons was studied. Of 11 monoclonal antibodies, 1 bound to all fragments and to both interferons, 7 bound to all fragments but not to the intact interferons, and 3 bound only to the 56-amino-acid fragment. In this latter study, the results may not directly address the issues under study here because the 56-amino-acid fragment represents about one-third of the interferon molecule and, thus, may be sufficiently large to be stably folded.

Several possible models may be proposed that are consistent with the observed high frequencies with which anti-peptide antibodies react with intact proteins. These models may be divided into two groups: those that bias the frequency of occurrence or recognition of native-like conformers and those that relax the constraint that the peptide must adopt a defined structure. The proposed models are not mutually exclusive.

There are several mechanisms by which the occurrence or the recognition of the native conformer may be biased. (*i*) The immune system may recognize preferentially the native conformation of the peptide. Although this seems to be highly unlikely at first consideration, the proteins investigated in this study (and indeed most if not all protein antigens) coevolved with the immune system, and preexposure to these or to related proteins is, in fact, possible, particularly in the case of the viral antigens. (*ii*) The receptors that trigger the immune response may "fix" one of the conformers of the peptide (analogous to the "induced-fit" model for substrate binding to enzymes). This is similar to the proposal by Kabat, who suggested that, for anti-polysaccharide antibodies, the antibody-binding might exert a selective effect on a relatively more flexible hexasaccharide antigen, which could adjust itself to the structure that best fit the combining site (22). Again the observed frequencies would require a predisposition of the immunoglobulin receptors toward native-like conformations. This problem differs from the possibility of induced fit in systems with a limited number of receptors (i.e., peptide hormones) because the immune system is a system of diversity, and unless other factors pertain (see above), "fixing" the correct conformation would seem to have no better probability than fixing the incorrect conformation. (*iii*) Factors involved in the presentation of the peptide antigens may restrict the range of conformations available to the peptides, making the native conformer far more common than predicted for the free peptide in aqueous solution. For example, the membranes of presenting cells may provide an environment that limits peptide conformation. The peptides in the present study were coupled to the carrier protein KLH, and interaction of the peptide with the surface of the carrier could be expected to greatly restrict the conformations accessible to the peptide. (*iv*) Peptides could have a more stable native-like structure that has been suggested from previous theoretical and experimental studies with model peptides. For example, peptides may contain particularly stable local structures like those shown by others (17–20). Stable local structures could serve as initiation sites in the folding of nascent or denatured proteins.

If true, such a model would have important implications for the general problem of protein folding (23–36).

Two related models, which relax the constraint that the peptide must adopt a defined local native-like conformation, may be proposed. Implicit in these models is the possibility that the free peptide and the peptide in the intact protein are subject to induced fit upon binding to the receptor or effector antibody molecule. These models deal only with local perturbations of protein structure because other studies show that anti-peptide antibodies can neutralize viruses (37) or inhibit enzyme activities (refs. 4, 38, and 39; unpublished data), suggesting that such antibodies recognize properly folded molecules.

The first model suggests that, for some percentage of the time, local disorder occurs on short segments of the protein, allowing reaction with anti-peptide antibodies. This is similar to the model suggested by Furie *et al.* (40), who used a fragment of Staphylococcal nuclease (residues 99–149) to study the relative ability of anti-fragment antibodies to bind to the intact protein. Although the antibodies were initially made to the fragment of residues 99–149, they were further fractionated so that only those that recognized the fragment of residues 99–126 were used. They found that it took about a 3,000-fold molar excess of native nuclease to displace 50% of the bound fragment from the antibody. They interpreted this to mean that only 0.03% of the native nuclease molecules are sufficiently unfolded to be recognized by antibodies specific for the fragment, which they presumed to be randomly folded. This calculation is suspect, however, because it assumes that the fragment is randomly folded, and it depends heavily on the relative affinity of the antibodies for the fragments and for the unfolded protein. This local disorder model suggests that peptides that elicit antibodies recognizing intact proteins are located in areas of relatively great conformational mobility in the intact protein.

In the second model, the condition that a peptide generate a self-recognizing antibody selects for peptides in which a large percentage of the conformers have a similar disposition of side chains and main-chain functional groups that are recognized by the immune system. Such would be the case for a peptide in which the limiting recognition site is in an extended conformation in a high percentage of the conformers. The high frequency of crossreaction with the intact protein then would reflect the frequency with which the limiting site is in a similar (not necessarily identical) conformation in the intact protein. Indeed, Peterfy *et al.* have shown that for monoclonal antibodies there may be a range of affinities that can be reflected in the differential sensitivities of methods of assay (41). The frequency of crossreaction suggests also that the limiting site is comprised of relatively short stretches of contiguous amino acids. As in the previous model, the frequency of crossreaction is explained most easily if the recognition site is one of relatively high flexibility in the intact protein.

Although we currently are unable to determine which of these models (or other models that may have escaped our attention) is correct, the availability of the frequency estimates has made the stochastic model less likely and forces consideration of other models, which may be tested by further studies including spectroscopic experiments (especially NMR) and crystallographic experiments. Finally, aside from theoretical considerations, the ability to generate easily monoclonal anti-peptide antibodies that react with intact proteins allows one to combine the purity of monoclonal reagents with the predetermined nature of anti-peptide antibodies to generate immunological tools of unprecedented specificity.

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